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Cytotoxic and Growth Inhibitory Effect of Ascorbic Acid on Cultured Bovine Vascular Endothelial Cells

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ABSTRACT—Ascorbic acid (AsA) is highly concentrated in vitreous in bovine, human as well as in other species. In order to evaluate the role of ascorbic acid as an ocular neovascularization inhibitor, we examined the effect of ascorbic acid on growth and survival of cultured vascular endothelial cells. When added to culture medium, high concentration of ascorbic acid (0.3 mM<) reduced viability of bovine aortic endothelial cells (BAEC) within 24 hr. Morphology of ascorbic acid treated endothelial cells demonstrated that fragmentation of nuclei does not accompany during this incubation period, suggesting that ascorbic acid induces vascular endothelial cell death in a non-apoptotic manner. To further confirm that this event was not specific on BAEC, bovine retinal endothelial cells (BREC) and human aortic endothelial cells (HAEC) were tested for ascorbic acid cytotoxicity. Ascorbic acid induced cell death in all three types of cells, but the dose required for induction of cell death differed, human endothelial cells were apparently more resistant to ascorbic acid cytotoxicity than bovine cells. Decrease in viability of BAEC exposed to ascorbic acid were partially inhibited by exposure to low oxygen concentration (O_2 = 1%). Addition of vascular endothelial growth factor (VEGF) stimulated proliferation of both BAEC and BREC, but co-addition of ascorbic acid reduced VEGF-induced endothelial cell proliferation. These results show that ascorbic acid modulates endothelial cell behavior *in vitro* and suggest that it is a negative regulator for ocular neovascularization.

INTRODUCTION

Fully developed mammalian vitreous is normally maintained avascular by neovascularization inhibitory factors (Preis *et al*., 1977; Raymond *et al*., 1982; Lutty *et al*., 1985; Jacobson *et al*., 1985). But in sites of proliferative diabetic retinopathy, augmentation of stimulatory factors exceeds the inhibitory effects, and proliferation and migration of retinal vascular endothelial cells followed by neovascularization leads to retinal disorders and visual loss. Factors modulating endothelial cell growth in sites of retinal ischemia has been identified, vascular endothelial growth factor (VEGF) which expression is induced by hypoxic exposure in several types of retinal cells *in vitro* (Aiello *et al*., 1995) and elevated in vitreous, retina and choroid tissues *in vivo* (Adamis *et al*., 1994; Lutty *et al*., 1996) closely correlates with active neovascularization and is considered to play a key role in this event. On the contrary, factors inhibiting neovascularization have not been fully elucidated and requires further investigations.

We have recently identified that bovine vitreous humor reduces viability of bovine aortic endothelial cells, and this was due to the action of ascorbic acid. Ascorbic acid cytotoxicity and growth inhibitory effect has been reported on various types of cells including tumor cell lines (Kao *et al*., 1993; Leung *et al*., 1993), keratocytes (Saika, 1993) and fibroblasts (Peterkofsky *et al*., 1977; Jampel, 1990; Schmidt *et al*., 1993). In contrast, despite the fact that mammalian ocular tissues preserve high concentration of ascorbic acid (Rose and Bode, 1991), the physiological effect of ascorbic acid on endothelial cells and its relation to ocular neovascularization has not been focused upon. In the present report, in order to evaluate the effect of ascorbic acid as an ocular neovascularization inhibitor, we characterized cytotoxicity of ascorbic acid on vascular endothelial cells and further investigated its effect on VEGF-induced endothelial cell proliferation.

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MATERIALS AND METHODS

Endothelial cell cultures

Bovine aortic endothelial cells (BAEC) were isolated from bovine aorta with collagenase digestion according to the method of Macarak *et al.* (1977). Cells were cultured in Eagle's minimum essential medium (MEM) (Nissui Pharmaceutical, Tokyo, Japan) supplemented with 10% fetal bovine serum (FBS) (JRH Biosciences, Lenexa, KS) at 37°C in humidified atmosphere with 5% CO_2.

Bovine retinal endothelial cells (BREC) were isolated by a modification of the methods of Capetandes and Gerritsen (1990). Briefly, retinas were aseptically removed and placed in MEM and vortex mixed. The mixture was trapped on 59 μm nylon mesh, washed extensively with phosphate buffered saline (PBS), and the remaining retentate was digested in 0.066% collagenase (Worthington, CLS, Cooper,

Freehold, NJ) and 0.033% BSA (Sigma, St.Louis, MO) in PBS for 45 min. at 37°C. The suspension was subjected to centrifugation (1000 × *g*, 5 min, 4°C) and the pellet was resuspended in Dulbecco's modified Eagle's medium (DME) (Nissui) supplemented with 20% serum and 100 μg/ml heparin. Cells were cultured on fibronectin pre-coated dishes with human pooled serum (Sigma) for primary culture, and on collagen coated dishes (Iwaki Glass Co., Ltd, Tokyo, Japan) supplemented with FBS for subcultures. Cells were characterized by uptake of 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate acetylated low density lipoprotein.

Human aortic endothelial cells (HAEC) were purchased from Clonetics (San Diego, CA). Cells were cultured in Clonetics endothelial cell growth medium (EGM BulletKit, Clonetics).

Fluorescence microscopic analysis

BAEC (3×10^4cells/well) were cultured in MEM supplemented with 10% FBS on Labtek (Nunc-Inc, Napeville, IL). Following 24 hr of incubation, medium was exchanged to 300 μl serum-free medium, with or without ascorbic acid. Cells were incubated for 24 hr, and then washed with PBS twice, fixed with 70% ethanol and stained with 50 μl of 100 μg/ml propidium iodide. The specimens were observed under a Nikon microscope (E600 model, Nikon, Tokyo, Japan) equipped with fluorescein optics.

Cell viability assay

Vascular endothelial cells were plated onto 24 well plates at 4.5×10^4 cells/well and cultured in MEM supplemented with 20% FBS for 24 hr. Medium was exchanged to 1ml serum-free medium with ascorbic acid (0.05-2 mM). After 24 hr of incubation, cells were trypsinized and cell viability was determined with trypan blue dye exclusion test.

Hypoxic study

BAEC (7.5×10^3 cells/well) were plated onto 96 well plate and cultured for 24 hr. Medium was exchanged to 100 μl serum-free medium with ascorbic acid and exposed to 1% oxygen using an advanced computer controlled water-jacketed CO_2 incubator (BNR-110 model, Tabai Espec Corp., Osaka, Japan) with reduced oxygen control induced by nitrogen replacement. Cells incubated under standard normoxic conditions were used as control subjects (95% air/5% CO_2). Following 24 hr of incubation period cell viability was determined with alamar blue assays (Nakayama *et al.*, 1997). Cells were washed twice with PBS and changed to 10% alamar blue (BioSource International, Camarillo, CA) containing MEM with 1% FBS and the absorbance at 570 nm with 600 nm background subtraction was measured after 18 hr of incubation time. Cell viability was calculated against control cells incubated with medium only. Cell viability determined by alamar blue assay was previously confirmed to correspond with the number of viable cells with dye exclusion tests.

Cell proliferation assay

Bovine vascular endothelial cells (1×10^3cells/well) were seeded onto 96 well collagen-coated plates and cultured in MEM supplemented with 20% FBS for 24 hr. After washing twice with PBS, medium was exchanged to DME containing 0.25% FBS with 10 ng/ml human recombinant VEGF (Sigma) with ascorbic acid (0.1-0.5mM). Following 72 hr of culture period cells were trypsinized and cell numbers were determined using a Coulter Counter (model ZM, Coulter, Luton, UK).

Statistics

Determinations were performed in triplicate, and each experiment was repeated at least three times. Results are expressed as means ± SE and were analyzed using a nonpaired Student's *t* test.

RESULTS

Characterization of ascorbic acid induced bovine vascular endothelial cell death

To evaluate the effect of ascorbic acid on vascular endothelial cells, BAEC were cultured with ascorbic acid in a serum-free medium. As shown in Fig. 1, treatment with ascorbic acid significantly reduced endothelial cell viability within 24 hr. The dose 0.3 mM was critical for induction of BAE cell death, and at higher concentrations comprising the bovine vitreal level of 1 mM, ascorbic acid induced cell death in a similar manner (data not shown). To further characterize cell death induced by ascorbic acid, morphological changes of ascorbic acid treated endothelial cells were examined under phase-contrast microscopy (Fig. 2) and DNA staining with propidium iodide (Fig. 3). Morphology of ascorbic acid treated BAEC were contracting and detaching from the plate (Fig. 2B) as compared with that of non-treated cells (Fig. 2A). Nucleic staining of ascorbic acid treated cells demonstrated condensed nuclei, but no fragmented nuclei were observed either at 0.3 mM (Fig. 3C) or 1mM (Fig. 3D) of ascorbic acid treatment. Because 24 hr of exposure time is sufficient to reduce viability of endothelial cells down to 2% as demonstrated in Fig. 1, it is considered that ascorbic acid induces vascular endothelial cell death in a non-apoptotic manner within this incubation period. This was further confirmed by lack of DNA fragmentation or failure of cell death inhibition by cycloheximide (data not shown). Presence of EDTA in medium significantly inhibited BAE cell death even under the high dose of 1 mM ascorbic acid (Fig. 4). Therefore it is suggested that ex-

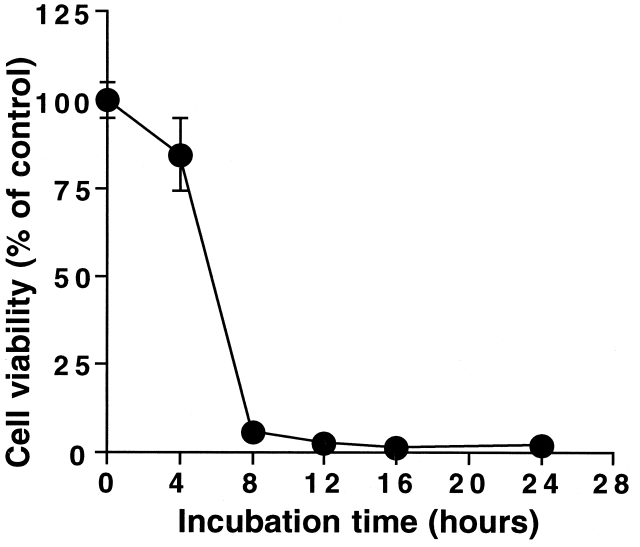

Fig. 1. Effect of ascorbic acid on cultured bovine aortic endothelial cells. Bovine aortic endothelial cells (BAEC) were seeded 4.5×10^4 cells/dish onto 24 well plates and cultured for 24 hr. Medium was changed to MEM with 0.3mM ascorbic acid, and after each indicated incubation times cells were trypsinized and viable cells were counted with trypan blue dye exclusion test. Cell viability was determined against control cells cultured for the same incubation period with medium only.

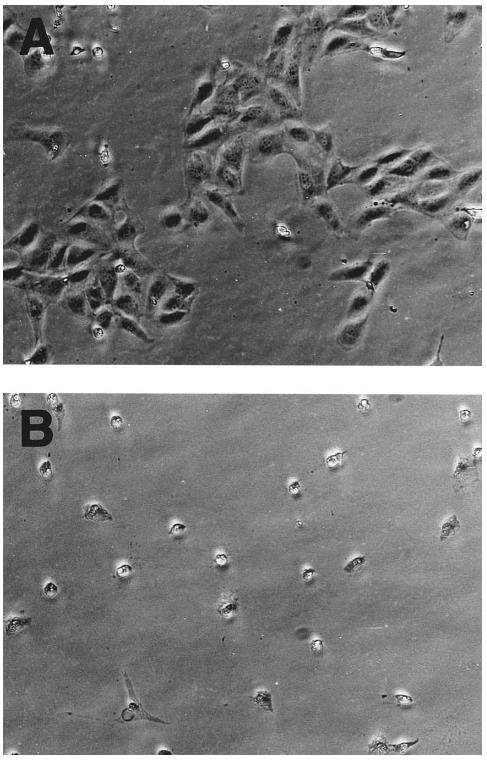

Fig. 2. Morphology of ascorbic acid treated BAEC. BAEC were seeded 2×10^5 cells/dish onto 35 mm dishes and cultured for 24 hr. Medium was changed to MEM with (B) or without (A) 0.3 mM ascorbic acid and after 24 hr of incubation photographs were taken under phase contrast microscopy.

tracellular Ca^{2+} is required in ascorbic acid induced cell death.

Susceptibility of vascular endothelial cells to ascorbic acid

To further confirm that this event was not specific on BAEC, BREC and HAEC were tested for ascorbic acid cytotoxicity. When exposed to ascorbic acid, all tested endothelial cells exhibited a dose-dependent decrease in viability (Fig. 5). However, the ascorbic acid concentration required for cell death varied between cell types (0.25 mM < BAEC, 0.5 mM < BREC, 1 mM < HAEC), HAEC were relatively resistant to ascorbic acid cytotoxicity as compared with bovine endothelial cells.

Effect of low oxygen concentration on ascorbic acid cytotoxicity

In sites of diabetic retinopathy, oxygen concentration drops to a low level due to retinal ischemia. Because this low level of oxygen concentration may alter susceptibility of endothelial cells to ascorbic acid, the effect of oxygen concentration on ascorbic acid cytotoxicity was investigated. When BAEC were exposed to ascorbic acid (0.2 mM) for 24 hr under normoxic (O_2 = 21%) condition, cell viability decreased to 13.7% (Fig. 6). Exposure to the same dose of ascorbic acid under hypoxic (O_2 = 1%) condition inhibited decrease in viability by 35.1% ($p < 0.05$). Oxygen concentration did not affect endothelial cell viability at low ascorbic acid concentration (0.1 mM), and at higher ascorbic acid doses (0.4 mM<)

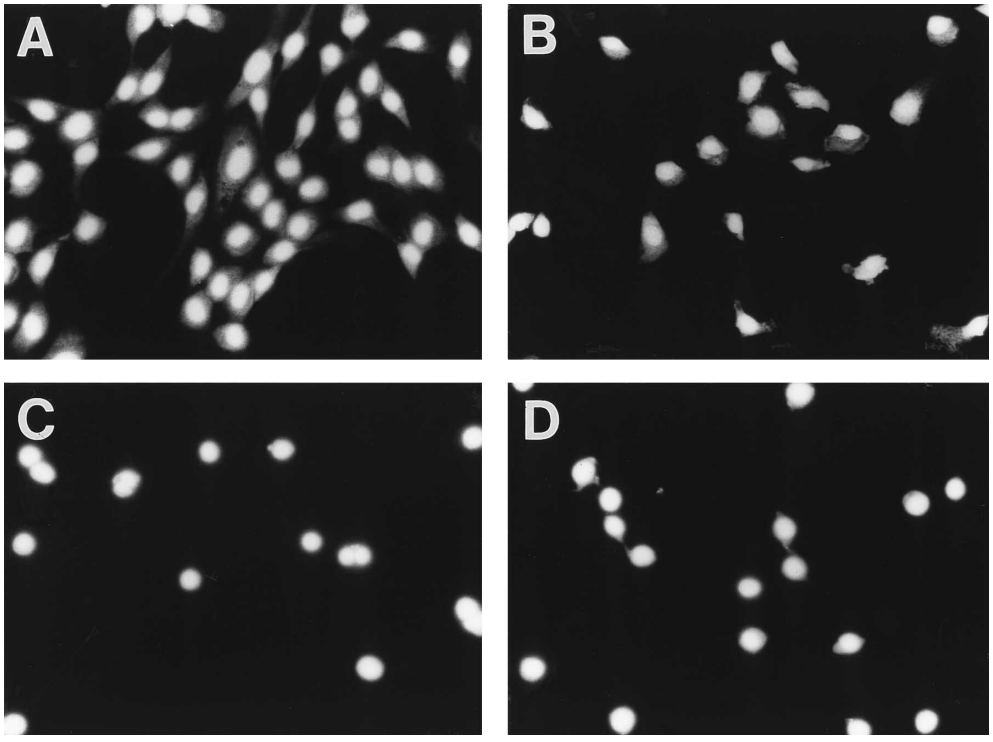

Fig. 3. DNA staining of ascorbic acid treated BAEC. BAEC were seeded onto chamber slides and prepared as described in the text. Figures show (A) control cells after 24 hr of incubation, (B) 6 hr after treatment with 0.3 mM ascorbic acid, (C) 24 hr after treatment with 0.3 mM ascorbic acid, (D) 24 hr after treatment with 1 mM ascorbic acid.

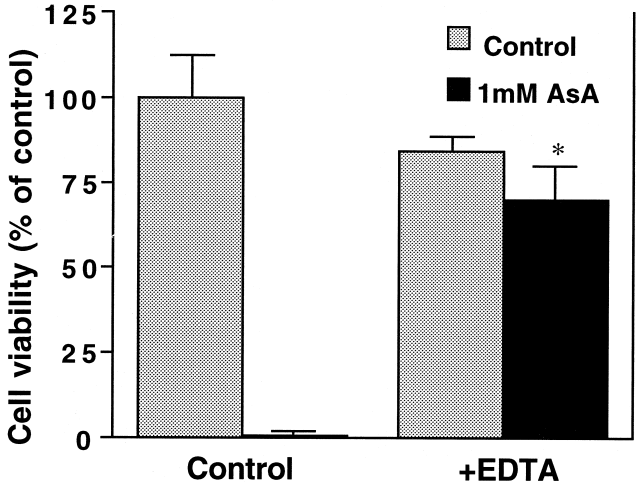

Fig. 4. Inhibition of ascorbic acid induced cell death by EDTA. BAEC were seeded 7.5×10^3 cells/well onto 96 well plates and incubated for 24 hr. Medium was changed to MEM with or without 25 μM EDTA in the presence or absence of 1 mM ascorbic acid. After 24 hr of incubation, cell viability was determined as described in the text. Cells cultured with MEM only for the same incubation period serves as control. *$P < 0.05$ with respect to 1 mM ascorbic acid treatment alone.

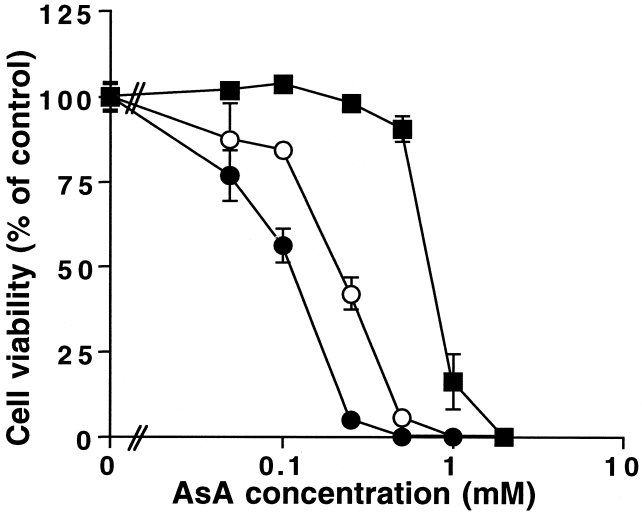

Fig. 5. Dose responsive curve of cultured vascular endothelial cells to ascorbic acid. Vascular endothelial cells were seeded 4.5×10^4 cells/dish onto 24 well plates and cultured for 24 hr. Medium was changed to MEM with ascorbic acid at the concentration indicated. After 24 hr of incubation, cells were trypsinized and viable cells were counted with trypan blue dye exclusion test. Cell viability was determined against respective endothelial cells cultured in the absence of ascorbic acid for the same incubation period. Symbols indicate; BAEC (●), BREC (○), HAEC (■).

BAE cell death was induced irrespective of the altering oxygen concentrations (data not shown).

Effect of ascorbic acid on VEGF-induced vascular endothelial cell proliferation

To investigate the effect of ascorbic acid on VEGF-induced endothelial cell proliferation, VEGF and ascorbic acid

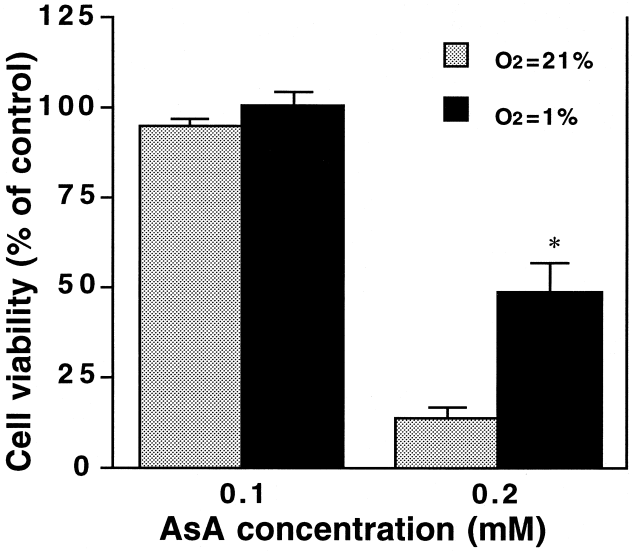

Fig. 6. Effect of low oxygen concentration on ascorbic acid cytotoxicity. BAEC were seeded 7.5×10^3 cells/well onto 96 well plates, and after 24 hr of incubation medium was changed to MEM with the indicated concentration of ascorbic acid. Cells were exposed to normoxic (O_2 = 21%) or hypoxic (O_2 = 1%) conditions for 24 hr and cell viability was determined against control cells cultured with MEM under normoxic condition. *$P < 0.05$ with respect to 0.2 mM ascorbic acid treatment under normoxic condition.

were co-added to the culture medium of BAEC and BREC. Treatment of endothelial cells with VEGF alone increased cell number by 122.0% and 116.4% in BAEC and BREC, respectively. Co-addition of ascorbic acid inhibited VEGF-induced endothelial cell proliferation in a dose-dependent manner under critical concentrations (BAEC 0-0.25 mM, BREC 0-0.5 mM) (Fig. 7). At higher doses (0.5 mM < BAEC, 1 mM < BREC), ascorbic acid induced cell death both in the presence or absence of VEGF (data not shown).

DISCUSSION

Although ascorbic acid cytotoxicity has been studied upon various types of cells, its effect on vascular endothelial cells has been poorly understood. In our present study we investigated the effect of ascorbic acid within the concentration in vitreous on cultured vascular endothelial cells. While ascorbic acid induces apoptosis in tumor cell lines (Sakagami *et al.*, 1996), our present studies demonstrate that vascular endothelial cell death induced by ascorbic acid differ from a characteristic apoptotic death. This is noteworthy because it has been reported that exposure to low concentration of hydrogen peroxide induces apoptotic cell death in BAEC (De Bono and Yang, 1995), and we have previously revealed that cytotoxic activity of ascorbic acid can be eliminated by catalase (unpublished data). Thus although hydrogen peroxide mediates ascorbic acid induced cell death, ascorbic acid induced endothelial cell death may not be consistent with that induced by hydrogen peroxide alone. Because oxygen free radical mediated necrotic death accompanies increase in cytosolic

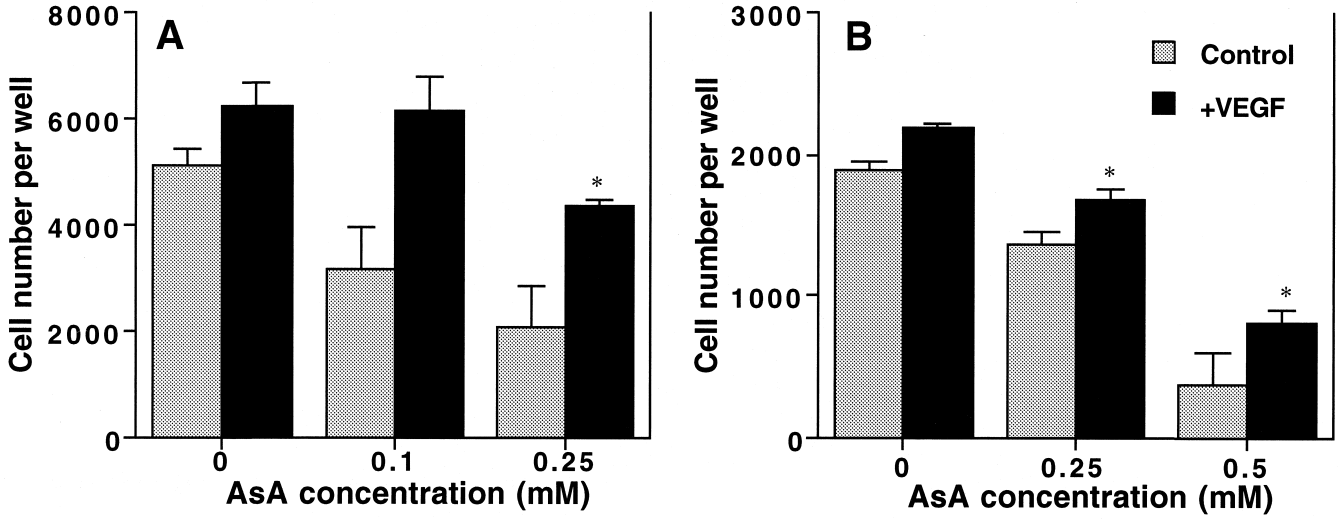

Fig. 7. Effect of ascorbic acid on VEGF-induced vascular endothelial cell proliferation. BAEC (A) and BREC (B) were seeded 1×10^3 cells/well onto 96 well plates. After 24 hr of incubation medium was changed to 0.25% FBS containing DME without (control) or with 10 ng/ml VEGF with ascorbic acid. After 72 hr of culture period cells were trypsinized and cell numbers were determined as described in the text. $*P < 0.05$ with respect to VEGF treated cells in the absence of ascorbic acid.

free Ca^{2+} which can be prevented by removal of extracellular Ca^{2+} (Geeraerts *et al.*, 1991), our findings that ascorbic acid induced BAE cell death is inhibited by EDTA suggest that it shares a common pathway with oxidative stress induced cell death.

We have also demonstrated that ascorbic acid induces cell death in vascular endothelial cells derived from different sources. It is interesting that HAEC requires higher dose of ascorbic acid for the induction of cell death, because ascorbic acid concentration in human vitreous is relatively high (2 mM) as compared with that in bovine (0.8-1.4 mM) (Nordmann, 1968). Human vitreous may concentrate higher dose of ascorbic acid in order to inhibit neovascularization. In other words, slight decrease in vitreal ascorbic acid concentration may tolerate human endothelial cell survival and initiate its proliferation. Report that vitreal ascorbic acid concentration are less pronounced in proliferative diabetic retinopathy patient (Takano *et al.*, 1997) gives a supportive data for such assumptions. In order to investigate further the correlation between ascorbic acid and ocular neovascularization, the effect of low oxygen concentration and VEGF on ascorbic acid cytotoxicity was examined. Low oxygen concentration (O_2 = 1%) inhibited ascorbic acid cytotoxicity at critical ascorbic acid concentration, which gives possibilities that concurrent decrease in ascorbic acid and oxygen level during diabetic retinopathy may tolerate endothelial cell survival *in vivo* as well. Co-addition of VEGF and ascorbic acid on vascular endothelial cells revealed that ascorbic acid reduces VEGF-induced endothelial cell proliferation in a dose-dependent manner. This was additive under critical ascorbic acid concentrations and at higher doses ascorbic acid induced cell death irrespective of the presence of VEGF. Thus although VEGF alone can stimulate endothelial cell proliferation, reduction of ascorbic acid concentration is considered essential for its effect, and moreover, vascular endothelial cell survival. In conclusion, our studies demonstrated that ascorbic acid modulates endothelial cell behavior *in vitro* and suggest that it is a negative regulator for ocular neovascularization.

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